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# **Developmental regulation of hippocampal long-term depression by cofilin-mediated actin reorganization**

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## **Keywords**

Hippocampal LTD, AMPA receptor, NSF, PICK1, Actin, Cofilin, Developmental regulation

## **Abstract**

Long lasting synaptic plasticity involves both functional and morphological changes, but how these processes are molecularly linked to achieve coordinated plasticity remains poorly understood. Cofilin is a common target of multiple signaling pathways at the synapse and is required for both functional and spine plasticity, but how it is regulated is unclear. In this study, we investigate whether the involvement of cofilin in plasticity is developmentally regulated by examining the role of cofilin in hippocampal long-term depression (LTD) in both young (2 weeks) and mature (2 months) mice. We show that both total protein level of cofilin and its activation undergo significant changes as the brain matures, so that although the amount of cofilin decreases significantly in mature mice, its regulation by protein phosphorylation becomes increasingly important. Consistent with these biochemical data, we show that cofilin-mediated actin reorganization is essential for LTD in mature, but not in young mice. In contrast to cofilin, the GluA2 interactions with NSF and PICK1 appear to be required in both young and mature mice, indicating that AMPAR internalization is a common key mechanism for LTD expression regardless of the developmental stages. These results establish the temporal specificity of cofilin in LTD regulation and suggested that cofilin-mediated actin reorganization may serve as a key mechanism underlying developmental regulation of synaptic and spine plasticity.

## **1. Introduction**

Plastic changes at glutamatergic synapses are critically important for both brain development and function, and deficits in these changes are responsible for a wide range of neurodevelopmental and neuropsychiatric disorders. Long-lasting synaptic plasticity, including long-term potentiation (LTP) and depression (LTD), a key mechanism for memory formation, involves changes in both synaptic

morphology and electrical transmission (also referred to as morphological and functional plasticity respectively) (Bliss and Collingridge, 1993; Malenka and Bear, 2004; Lamprecht and LeDoux, 2004; Alvarez and Sabatini, 2007; Bourne and Harris, 2008). Although extensive studies exist to elucidate the molecular mechanisms underlying these two forms of plasticity individually, how these changes interact and are coordinated during synaptic remodeling remains poorly understood.

In the CA1 region of the hippocampus, where the molecular mechanisms underlying LTP and LTD are most extensively studied, the induction of both functional and morphological changes requires activation of NMDA receptors (NMDARs) and subsequent  $\text{Ca}^{2+}$ -dependent signaling processes (Bliss and Collingridge, 1993; Malenka and Bear, 2004; Lamprecht and LeDoux, 2004; Alvarez and Sabatini, 2007; Bourne and Harris, 2008). Although AMPA glutamate receptors (AMPA receptors), the principal mediator of fast excitatory synaptic transmission, are a major target of these signaling processes that express and maintain functional changes (Malinow and Malenka, 2003; Brecht and Nicoll, 2003; Collingridge et al., 2004; Malenka and Bear, 2004; Shepherd and Huganir, 2007; Lüscher and Huber, 2010; Collingridge et al., 2010), the actin cytoskeleton, the predominant structural component of the dendritic spine, is believed to be the main determinant that governs morphological plasticity (Luo et al., 2002; Cingolani and Goda, 2008; Asrar and Jia, 2013). In this regard, we and others have previously shown that Rho GTPase-activated protein kinases, including p21-activated kinases (PAK1 and 3), Rho-kinase 2 (ROCK2) and LIM-domain containing kinase (LIMK1), are critically involved in both LTP, LTD and associated spine plasticity (Meng et al., 2002, 2004; Meng et al., 2005; Asrar et al., 2009; Zhou et al., 2009; Huang et al., 2011; Bosch et al., 2014; Todorovski et al., 2015; Nishiyama and Yasuda, 2015). Importantly, these kinases all target cofilin, a potent regulator of the actin cytoskeleton (Bernstein and Bamburg, 2010), suggesting that cofilin-mediated actin reorganization may serve as a common effector to mediate,

and potentially coordinate functional and morphological changes during synaptic remodeling (Meng et al., 2003a; Jia et al., 2009; Asrar and Jia, 2013; Rust, 2015). However, how cofilin is regulated during plasticity remains largely unknown.

In this study, we investigated whether cofilin is regulated during mouse development, a process characterized by profound changes in both spine dynamics and functional plasticity, and if so, how this developmental regulation impacts its involvement in hippocampal LTD. We provide evidence that both cofilin expression/activity and its involvement in hippocampal LTD change dramatically as brain matures, suggesting that cofilin-mediated actin reorganization may serve as a key mechanism underlying developmental regulation of synaptic plasticity.

## **2. Methods**

### *2.1. Electrophysiology*

Electrophysiological recordings were conducted at the Schaffer/Collateral pathway in the hippocampus as previously described (Meng et al., 2002, 2003; Zhou et al., 2011). Briefly, the mouse brains of either sex were quickly removed and sagittal 300-400  $\mu\text{m}$  hippocampal slices prepared in ice-cold artificial cerebrospinal fluid (ACSF) saturated with 95%  $\text{O}_2$ /5%  $\text{CO}_2$ . ACSF contained (in mM): 120 NaCl, 3.0 KCl, 1.2  $\text{MgSO}_4$ , 1.0  $\text{NaH}_2\text{PO}_4$ , 26  $\text{NaHCO}_3$ , 2.0  $\text{CaCl}_2$ , and 11 D-glucose. These acute slices were recovered at 22~26°C for at least 2 hours, then transferred to a submersion chamber perfused with 95%  $\text{O}_2$ /5%  $\text{CO}_2$  saturated ACSF with (for whole-cell recordings) or without (for field recordings) 100 $\mu\text{M}$  picrotoxin. Perfusion flow rate was set at 2ml/min. Hippocampal CA1 neurons were visualized using an infrared differential interference contrast microscope (Zeiss Axioscope or Olympus X51). Synaptic transmission was evoked at

0.05 Hz for field recordings and 0.1 Hz for whole-cell recordings and recorded with glass pipettes (3-4 M $\Omega$ ) filled with either ACSF (for field responses) or the intracellular solution (for whole-cell response) containing (in mM) 130 CsMeSO<sub>4</sub>, 5 NaCl, 1 MgCl<sub>2</sub>, 0.05 EGTA, 10 HEPES, 3 Mg-ATP, 0.3 Na<sub>3</sub>GTP, and 5 QX-314 (pH 7.25) (280-300 mOsm). For whole-cell experiments, cells were clamped at -65 mV throughout the experiment. Whole-cell series resistance was monitored throughout LTD experiments by applying a -3 mV step at the end of each response sweep and the experiment was excluded from analysis if resistance changed by more than 20%. For peptide infusion experiments, the lack of the effect of each peptide on basal synaptic responses was independently tested by conducting baseline recordings in the presence of the peptides for at least 1 hour without LTD induction. Mice at the age of 2 weeks were defined as 'young mice' and 2-3 months 'mature mice'. In all recordings using peptides or chemicals, control and experimental groups were tested alternately to minimize variables. Field EPSP LTD induction protocols used in this study were: low-frequency stimulation (LFS, 900 pulses at 1Hz), paired-pulse LFS (PP-LFS, 900 pairs of pulses at 1Hz with 50 ms pairing interval), NMDA chemical LTD (25  $\mu$ M for 3 min) and DHPG chemical LTD (100  $\mu$ M for 10 min). LTD of whole-cell EPSC recordings was induced by 300 pulses at 1Hz (LFS) or 600 pairs of pulses at 1Hz with 50 ms pairing interval (PP-LFS) or 100  $\mu$ M DHPG perfusion for 10 min. Data acquisition and analysis were done using pClamp 10 software (Molecular Devices, USA). N in all figures represents the number of slices and at most two slices from each animal were used. All recording data were statistically evaluated with Student's *t*-test.

## *2.2. Slice treatment and biochemical assays*

In order to correlate electrophysiological and biochemical data, protein lysates extracted from acutely prepared hippocampus were analyzed. The conditions for preparing and maintaining hippocampal slices were the same as for electrophysiological recordings. For each experiment, slices were recovered for at least 2 hours at room temperature in 95% O<sub>2</sub>/5% CO<sub>2</sub> saturated ACSF, then transferred to a treatment chamber for additional 30 min recovery before DHPG treatment. The treatment experiments were divided into two groups: slices removed immediately before DHPG application were used as untreated control and slices treated with 100  $\mu$ M DHPG for 10 minutes as DHPG treated group. Hippocampal slices were frozen in dry ice/ethanol slurry and stored at -20 °C at the end of each treatment. Samples were lysed for 50 minutes in ice-cold lysis buffer containing (in mM) 20 Tris pH 7.5, 150 NaCl, 1 EDTA, 1 EGTA, 1% Triton X-100, 2.5 sodium pyrophosphate, 1  $\beta$ -glycerophosphate, 1 Na<sub>3</sub>VO<sub>4</sub>, 20 NaF, 1  $\mu$ g/ml leupeptin, 1 PMSF, and 0.5% protease inhibitor cocktail (Calbiochem) and phosphatase inhibitor (Roche). The supernatant was collected by centrifugation at 12,000 rpm (4°C) for 10 minutes. For synaptosomal fraction preparation, Hippocampi were isolated, homogenized 0.32M sucrose (in HBSS) and transferred to polycarbonate centrifuge tube for centrifugation at 1,000g, 4°C for 10 minutes to remove the nuclear fraction. The supernatant (S1) was collected and centrifuged at 13,800g for 10 minutes to separate supernatant (S2) and pellet (P2) that contains microsomes/light membranes and crude synaptosomal fractions respectively. The P2 fraction was suspended in HBSS and subjected to discontinuous sucrose gradient (1.2M, 1.0M and 0.85M in HBSS w/o sucrose) centrifugation at 82,500g for 2 hr at 4°C. The synaptosomal fraction was then collected from the 1.0M to 1.2M gradient and subsequently centrifuged at 150,000g for 30 min to obtain the synaptosomal pellet. The pellet was suspended overnight at 4°C in sample buffer containing (in mM) 50 HEPES, 2 EDTA, 0.5% Triton, 20 NaF, 1 NaVO<sub>4</sub>

and 0.5% protease inhibitor cocktail (Calbiochem) and phosphatase inhibitor (Roche). The enrichment of synaptic proteins in synaptosomal fractions was verified using synaptic markers synapsin and PSD95. The total protein concentration of each sample was estimated by a BCA assay. For each experiment, protein samples (10 µg of total proteins) of each group were loaded on SDS gel. Proteins were separated on 12% SDS-PAGE separation gel and electrotransferred to a nitrocellulose filter. Filters were then blocked with 2% dry milk or 2% fetal bovine serum in TBST (20 mM Tris base, 9% NaCl, 0.1% Tween-20, pH 7.6) and incubated overnight at 4°C with cofilin or p-cofilin antibodies in TBST. After washing and incubated with appropriate secondary antibodies, membranes were extensively washed with TBS and subjected to chemiluminescence signal detection using a Pierce HRP kit. Band intensity was analysed using the AlphaEaseFC software. GAPDH was used as the protein loading control. Primary antibodies included anti-cofilin (CST), anti-p-cofilin (CST), anti-ADF (Sigma) and anti-GAPDH (Santa Cruz Biotechnology). N in the summary data represents the number of independent experiments.

### *2.3. Peptides and chemicals*

The mice (C57BL6) were housed and maintained under a standard 12/12 light/dark cycle condition. All the procedures used for this study were approved by the animal care committees at the Hospital for Sick Children, Canada and Southeast University, China. The drugs were purchased from Tocris (UK) and/or Sigma Aldrich (Canada and China). Active peptides pepEVKI (YNVYGIEEVKI) that blocks the GluA2-PICK1 interaction and its control pepSVKE (YNVYGIESVKE), active pep2m (KRMKVAKNAQ) that blocks GluA2-NSF interaction and its control pep4c (KRMKVAKSAQ), were purchased from Tocris (UK). Peptides S3 (MASGVAVSDGVIKVFN) that inhibits cofilin phosphorylation and pS3



(MASpGVAVSDGVIKVFN) that inhibits cofilin dephosphorylation were synthesized by APTC peptide synthesis facility (The Hospital for Sick Children, Canada) and GenScript (China). 100X peptide stock aliquots were added to the intracellular solution immediately before recording experiments.

### 3. Results

#### *3.1. Both protein level and activity of cofilin is developmentally regulated*

Cofilin is a potent regulator of actin reorganization, and its activity is stringently regulated by protein phosphorylation at serine3, primarily by LIMK1/2 (Arber et al., 1998; Yang et al., 1998; Bamburg, 1999; Govek et al., 2005; Bernard, 2007). We and others have previously shown that cofilin-dependent actin changes are required for spine regulation and hippocampal plasticity, in particular LTD (Meng et al., 2002; Jia et al., 2009; Rust et al., 2010; Huang et al., 2011; Zhou et al., 2011; Eales et al., 2014; Todorovski et al., 2015; Liu et al., 2016), but whether its involvement in these processes is developmentally regulated remains unknown. This is an important question because LTD is strongly affected by the developmental stages of the brain (Kemp et al., 1999; 2000; Fitzjohn et al., 2001). To investigate this issue, we analyzed total and phosphorylated (inactive) form of cofilin (p-cofilin) in both young (2 weeks) and mature (2 months) mice using antibodies specific to total and phosphorylated cofilin respectively. As shown in Fig. 1A, the total protein level of cofilin was much lower in mature compared to young mice ( $0.44 \pm 0.05$ ,  $n=16$ ,  $p<0.001$ ). Interestingly, the level of p-cofilin was also lower in mature compared to young mice ( $0.31 \pm 0.05$ ,  $n=14$ ,  $p<0.001$ ). Analysis of the ratio of p-cofilin vs cofilin indicated that the relative amount of p-cofilin per unit cofilin was significantly lower in mature mice (Fig. 1B, p-cofilin/cofilin:  $0.59 \pm 0.06$ ,  $n=14$ ,  $p<0.001$  compared with young mice). These

results suggest that although young mice have high levels of cofilin expression, which may underlie a more dynamic nature of the synapse and spine at this age, its regulation by protein phosphorylation/dephosphorylation may play a more pronounced role at the more stable, mature synapse, due to limited amount of total cofilin expressed at this age. Rapid activation and inactivation of cofilin by protein dephosphorylation and phosphorylation are consistent with its role in synaptic plasticity that requires rapid changes in the actin cytoskeleton in response to neuronal activities.

Because the protein lysis conditions that we used in above experiments were relatively mild (1% Triton), it was possible that some cofilin might be associated with insoluble materials in the pellet that was discarded during protein extraction, and this portion of cofilin might contribute to the differences in cofilin between young and mature mice. To test this possibility, we also analyzed the pellet fraction, but the results were similar to those obtained from the soluble fraction (Fig. 1C, cofilin:  $0.30 \pm 0.04$ ,  $n=8$ ,  $p<0.001$ ; pcofilin:  $0.35 \pm 0.06$ ,  $n=8$ ,  $p<0.001$ ), indicating that the differences in cofilin level between young and mature are not likely due to its differential ability to be extracted or to be associated with actin filaments or other insoluble materials. To determine if these developmental changes in cofilin occurred at the synapse, we analyzed synaptosomal fractions and found that, similar to total hippocampal protein lysates, both cofilin and p-cofilin (Fig. 1D, cofilin:  $0.51 \pm 0.09$ ,  $n=5$ ,  $p<0.001$ ; pcofilin:  $0.26 \pm 0.04$ ,  $n=4$ ,  $p<0.001$ ) as well as p-cofilin/cofilin ratio (Fig. 1E,  $0.41 \pm 0.06$ ,  $n=5$ ,  $p<0.001$ ) were much lower in mature than in young mice. Interestingly, the level of ADF, another member of the ADF/cofilin family, showed no differences between young and mature mice in either total hippocampal lysates (Fig. 1F,  $0.95 \pm 0.07$ ,  $n=8$ ,  $p>0.05$ ) or synaptosomal fractions (Fig. 1G,  $0.86 \pm 0.07$ ,  $n=6$ ,  $p>0.05$ ), suggesting that this developmental regulation is specific to cofilin.

To directly investigate whether cofilin activation during plasticity is developmentally regulated, we analyzed the effect of the group I mGluR agonist DHPG (100  $\mu$ M, 10 min) on total and p-cofilin in both young and mature mice. As shown in Fig. 1H and I, although the amount of total cofilin was not affected by DHPG, p-cofilin was significantly decreased after DHPG treatment in both young (Fig. 1H, cofilin:  $0.94 \pm 0.07$ ,  $n=5$ ,  $p>0.05$  compared with untreated; p-cofilin:  $0.75 \pm 0.09$ ,  $n=6$ ,  $p<0.05$  compared with untreated) and mature mice (Fig. 1I, cofilin:  $1.08 \pm 0.06$ ,  $n=5$ ,  $p>0.05$  compared with untreated; p-cofilin:  $0.64 \pm 0.09$ ,  $n=5$ ,  $p<0.01$  compared with untreated). The ratio of p-cofilin vs cofilin (Fig. 1J) was also decreased after DHPG treatment in both young ( $0.80 \pm 0.07$ ,  $n=5$ ,  $p<0.05$  compared with untreated) and mature mice ( $0.60 \pm 0.08$ ,  $n=5$ ,  $p<0.01$  compared with untreated). Interestingly, the degree of changes in both p-cofilin and the ratio of p-cofilin vs cofilin was more significant in mature than in young mice, suggesting that cofilin regulation by protein phosphorylation and dephosphorylation may play an increasingly more important role at the mature synapse.

### ***3.2. LTD can be induced in both young and mature hippocampus by using different protocols***

To investigate developmental regulation of cofilin in hippocampal LTD, we first established protocols to induce LTD reliably and consistently in both young and mature mice. As shown in Fig. 2A, low frequency stimulation (LFS, 900 pulsed at 1Hz), which is known to induce NMDAR-dependent LTD (NMDAR-LTD), elicited a consistent and long-lasting depression in young mice ( $73.7 \pm 5.7\%$ ,  $n=6$ ,  $p<0.05$  compared with baseline), but this LFS protocol failed to induce a sustained depression in slices obtained from mature hippocampus ( $102.6 \pm 5.4\%$ ,  $n=6$ ,

$p > 0.05$  compared with baseline). In contrast, as shown in Fig. 2B, the delivery of paired-pulse LFS (PP-LFS, 900 paired pulses at 1Hz with a 50 ms inter-pulse interval), readily induced LTD in both young ( $90.7 \pm 3.1\%$ ,  $n=9$ ;  $p < 0.05$  compared to baseline) and mature hippocampus ( $79.1 \pm 3.8\%$ ,  $n=9$ ;  $p < 0.05$  compared with baseline).

Because PP-LFS may induce both NMDAR-LTD and mGluR-dependent LTD (mGluR-LTD), we also tested whether LTD could be chemically induced by application of specific agonists for NMDARs or mGluRs. To induce NMDAR-LTD, we used a brief (3 min) bath application of 25  $\mu$ M NMDA. As shown in Fig. 2C, this form of LTD was obtained in both young and mature mice (young:  $44.3 \pm 2.7\%$ ,  $n=5$ ; mature:  $79.5 \pm 3.6\%$ ,  $n=7$ ;  $p < 0.05$  compared with baseline). To induce mGluR-LTD, we used bath application of 100  $\mu$ M DHPG (10 min). As shown in Fig. 2D, LTD was also obtained in both young and mature mice (young:  $72.3 \pm 6.5\%$ ,  $n=11$ ; mature:  $82.1 \pm 3.8\%$ ,  $n=7$ ;  $p < 0.05$  compared with baseline). Taken together, these results indicate that with appropriate induction protocols, LTD can be induced in both young and mature hippocampus. The establishment of these protocols had enabled us to test the role of cofilin and other proteins in LTD regulation in both young and mature mice.

### ***3.3. NSF and PICK1 are required for LTD in both young and mature hippocampus***

Before we examined the role of cofilin, we first determined whether the LTD that we induced was mechanistically similar to that previously characterized. Although NMDAR- and mGluR-LTD have distinct induction mechanisms, both appear to require the removal of AMPARs at the synapse (Malinow and Malenka, 2003; Brecht and Nicoll, 2003; Collingridge et al., 2004, 2010; Lüscher and Huber, 2010). In particular, protein interactions at the GluA2 C-terminal domain with NSF and PICK1 are critical for AMPAR internalization and expression of LTD (Nishimune

et al., 1998; Song et al, 1998; Noel et al., 1999; Luthi et al., 1999; Luscher et al., 1999; Xia et al., 1999; Daw et al., 2000; Kim et al., 2001; Braithwaite et al., 2002; Lee et al, 2002; Seidenman et al., 2003; Terashima et al., 2008; Jo et al., 2008; Citri et al., 2010). However, whether the involvement of these protein interactions is developmentally regulated is unknown. To address this issue, we utilized the GluA2 peptides that specifically disrupt GluA2-NSF and GluA2-PICK1 interaction respectively in both young and mature hippocampal slices. First, we tested the role of the GluA2-NSF interaction using the peptide pep2m that blocks the GluA2-NSF interaction and its control peptide pep4c. Consistent with previous reports (Nishimune et al., 1998; Luthi et al., 1999), the control peptide pep4c had no effect on either basal synaptic transmission (Fig. 3A, Ctrl:  $98.4 \pm 7.5\%$ ,  $n=5$ ,  $p>0.05$  compared to the first 10 min of the recordings) or LTD (Fig. 3A, LFS:  $67.4 \pm 4.1\%$ ,  $n=5$ ,  $p>0.05$  compared to the LTD without any peptide) in young mice. The control peptide also showed no effect in mature mice (Fig. 3B, Ctrl:  $100.2 \pm 6.1\%$ ,  $n=8$ ,  $p>0.05$  compared to the first 10 min of the recordings; PP-LFS:  $54.2 \pm 10.7\%$ ,  $n=5$ ,  $p>0.05$  compared to the LTD without any peptide). In contrast, pep2m gradually depressed basal synaptic transmission (Fig. 3C, Ctrl:  $69.0 \pm 4.5\%$ ,  $n=5$ ,  $p<0.05$  compared to responses at the beginning of the recordings or responses with pep4c). Interestingly, pep2m plus LFS depressed synaptic responses to a similar degree as pep2m alone (Fig. 3C, LFS:  $75.3 \pm 5.4\%$ ,  $n=7$ ;  $p>0.05$  compared with Ctrl). In mature mice, the active peptide pep2m also depressed basal synaptic transmission (Fig. 3D, Ctrl:  $80.1 \pm 5.4\%$ ,  $n = 8$ ) to a similar degree as pep2m plus PP-LFS (Fig. 3D, PP-LFS:  $81.4 \pm 7.0\%$ ,  $n = 5$ ;  $p > 0.05$  compared to Ctrl). These results indicate that in both young and mature mice pep2m induces synaptic depression that occluded LTD. To further investigate this, we attempted to induce LTD after the pep2m-induced synaptic depression had stabilized (i.e. 30 min after the infusion of the peptide). As shown in Fig. 3E, PP-LFS induced no

further depression (40-50 min:  $74.4 \pm 5.6\%$ , 90-100 min:  $69.2 \pm 8.1\%$ ,  $n = 6$ ;  $p > 0.05$ ), supporting that GluA2-NSF interaction is required for both basal synaptic transmission and LTD expression.

To determine the role of the GluA2-PICK1 interaction, we utilized the peptide pepEVKI that blocks the GluA2-PICK1 interaction and its control pepSVKE (Daw et al., 2000). As shown in Fig. 4, while the infusion of the control peptide pepSVKE had no effect on either basal synaptic response or LFS-induced LTD in both young (Fig. 4A, Ctrl:  $101.9 \pm 3.3\%$ ,  $n=5$ ;  $p>0.05$  compared to the first 10 min of the recordings; LFS:  $68.3 \pm 2.9\%$ ,  $n=6$ ;  $p<0.05$  compared to the first 10 min of the recordings and  $p>0.05$  compared to the LTD without any peptide ) and mature mice (Fig. 4B, Ctrl:  $97.1 \pm 5.0\%$ ,  $n=6$ ;  $p>0.05$  compared to the first 10 min of the recordings; PP-LFS:  $75.6 \pm 4.7\%$ ,  $n=5$ ;  $p<0.05$  compared to the first 10 min of the recordings and  $p>0.05$  compared to the LTD without any peptide), the active peptide pepEVKI significantly attenuated LTD without affecting baseline response in both young (Fig. 4C, Ctrl:  $96.6 \pm 2.1\%$ ,  $n=6$ ;  $p>0.05$  compared to the first 10 min of the recordings; LFS:  $88.2 \pm 2.3\%$ ,  $n=10$ ;  $p>0.05$  compared to the first 10 min of the recordings) and mature mice (Fig. 4D, Ctrl:  $95.3 \pm 5.8\%$ ,  $n=5$ ;  $p>0.05$  compared to the first 10 min of the recordings; PP-LFS:  $97.0 \pm 9.8\%$ ,  $n=4$ ;  $p>0.05$  compared to the first 10 min of the recordings). These results indicate that NSF- and PICK1-mediated AMPAR internalization is a common mechanism for LTD expression in both young and mature mice.

### ***3.4. Cofilin activation is required for LTD in mature, but not in young mice***

After we established that LTD requires NSF/PICK1 in both young and mature hippocampus, we then tested the role of cofilin-mediated actin dynamics at both ages. First, we examined the effect of actin depolymerization inhibitor phalloidin. As shown in Fig. 5A-C, whereas inclusion of of

100  $\mu$ M phalloidin to the recording pipette had no effect on LFS-LTD (Fig. 5A, Ctrl:  $63.0 \pm 7.2\%$ ,  $n=6$ ; phalloidin:  $62.3 \pm 5.9\%$ ,  $n=8$ ;  $p>0.05$ ) or PP-LFS-LTD (Fig. 5B, Ctrl:  $72.0 \pm 8.4\%$ ,  $n=5$ ; phalloidin:  $66.3 \pm 7.9\%$ ,  $n=7$ ,  $p>0.05$ ), it completely blocked PP-LFS-LTD in mature mice (Fig. 5C, Ctrl:  $65.3 \pm 8.0\%$ ,  $n=8$ ; phalloidin:  $98.6 \pm 8.4\%$ ,  $n=8$ ;  $p<0.05$ ). To determine if the effect of phalloidin is mediated by cofilin-dependent actin reorganization, we tested two cofilin peptides S3 and pS3 known to increase and decrease cofilin activity respectively (Aizawa et al., 2001; Huang et al., 2011). As shown in Fig. 5D, neither S3 nor pS3 peptides had an effect on LFS-LTD (S3:  $63.3 \pm 7.0\%$ ,  $n=7$ ; pS3:  $65.1 \pm 6.7\%$ ,  $n=5$ ;  $p>0.05$ ) in young mice. Similarly, neither peptides had an effect on PP-LFS-LTD (Fig. 5E), even when the concentration of pS3 was increased to 800  $\mu$ M (S3:  $62.9 \pm 4.4\%$ ,  $n=4$ ; pS3 at 200  $\mu$ M:  $45.8 \pm 5.2\%$ ,  $n=5$ ; high pS3 at 800  $\mu$ M:  $60.1 \pm 11.4\%$ ,  $n=7$ ;  $p>0.05$ ). However, pS3 peptide completely blocked PP-LFS-LTD in mature mice (Fig. 5F, S3:  $45.6 \pm 9.8\%$ ,  $n=6$ ; pS3:  $83.4 \pm 13.7\%$ ,  $n=5$ ;  $p<0.05$ ). These results indicate that, in clear contrast to NSF and PICK1, LTD in young mice, but not in mature is particularly sensitive to cofilin manipulations.

Because PP-LFS may induce both NMDAR- and mGluR-LTD, the inhibitory effect of cofilin and phalloidin in mature, but not in young mice, could be due to the differences in the relative contribution of these two forms of LTD, rather than a developmental effect. To address this issue, we analyzed the effect of cofilin specifically in mGluR-LTD induced by DHPG in both young and mature mice. Similar to LFS-LTD and PP-LFS-LTD, neither S3 nor pS3 had any effect on DHPG-induced LTD in young mice (Fig. 6A, S3:  $55.7 \pm 6.7\%$ ,  $n=9$ ; pS3:  $60.6 \pm 6.9\%$ ,  $n=7$ ;  $p>0.05$ ). In contrast, pS3 but not S3 completely blocked DHPG-induced LTD in mature mice (Fig. 6B, S3:  $55.9 \pm 6.7\%$ ,  $n=6$ ; pS3:  $95.3 \pm 6.3\%$ ,  $n=9$ ;  $p<0.05$ ). To confirm that this DHPG-induced LTD is also dependent on AMPAR internalization in both young and mature mice, we

again tested the effect of the peptide pepEVKI to disrupt GluA2-PICK1 interaction. As shown in Fig. 6C, while the infusion of the control peptide pepSVKE had no effect on DHPG-induced mGluR-LTD in young mice, the active peptide pepEVKI significantly attenuated LTD in these mice (Fig. 6C, SVKE:  $70.0 \pm 4.6\%$ ,  $n=7$ ; EVKI:  $88.2 \pm 5.5\%$ ,  $n=7$ ;  $p<0.05$ ). Importantly, the active, but not the control peptide also blocked DHPG-LTD in mature mice (Fig. 6D, SVKE:  $75.3 \pm 4.8\%$ ,  $n=8$ ; EVKI:  $93.3 \pm 6.2\%$ ,  $n=6$ ;  $p<0.05$ ). These results indicate that although AMPAR internalization is required for LTD expression in both young and mature mice, the involvement of cofilin-mediated actin reorganization is developmentally regulated, being essential in mature, but not in young hippocampus.

#### **4. Discussion**

Cofilin is a common target of the Rho family small GTPases (Bernstein and Bamburg, 2010) and is shown to be important for both spine morphology, receptor trafficking and synaptic plasticity (Meng et al. 2004; Jia et al., 2009; Gu et al., 2010; Rust et al., 2010; Bosch et al., 2014; Eales et al., 2014; Rust, 2015). In particular, cofilin is indispensable for the expression of hippocampal LTD and associated spine plasticity (Zhou et al., 2004; Rust et al., 2010; Zhou et al., 2011; Eales et al., 2014). However, whether the involvement of cofilin in LTD is regulated by developmental stages remains unknown. In this study, we systematically investigated the role of cofilin in LTD at hippocampal CA1 synapse in both young and mature mice. We show that both baseline cofilin and its activation by LTD protocols are developmentally regulated and importantly, its requirement in LTD expression is sensitive to the developmental stage. These results suggest that cofilin-mediated actin reorganization may play differential roles in different forms of plasticity during brain development.



First, we show that the protein level of cofilin, but not of ADF, is developmentally regulated. Thus, in young mice, the amount of cofilin is significantly higher (by 70%) than that in mature mice. The high level of cofilin at this stage of development suggests that the actin cytoskeleton is more dynamic, consistent with rapid neuronal growth and synaptogenesis in developing brain. Interestingly, the level of inactive cofilin (i.e. p-cofilin) also decreases dramatically (by 80%) as brain matures, suggesting that although the total amount of cofilin is restricted to a low level, which is consistent with more stable actin cytoskeleton at mature synapse, cofilin activation by protein dephosphorylation may become increasingly important in mediating actin reorganization. Indeed, we show that DHPG treatment does not induce changes in total amount of cofilin, but a significant decrease in p-cofilin and this DHPG-induced cofilin activation appears to be significantly more in mature than young mice. Taken together, these biochemical data suggest that rapid regulation of cofilin activity by protein phosphorylation and dephosphorylation may be particularly important during synaptic plasticity at mature synapse. It is important to note that the antibodies used in this study do not distinguish cofilin 1 and 2, therefore their specific contribution will require further investigations. However, because cofilin 1 is the predominant brain form (Bamburg, 1999; Rust et al., 2010), the effect on LTD is likely mediated by cofilin 1.

Second, we establish protocols to induce and characterize hippocampal LTD in both young and mature mice. Consistent with previous studies (Kemp et al., 1999; Fitzjohn et al., 2001) we show that while synaptic stimulation with LFS induces LTD only in young mice, PP-LFS is capable of inducing LTD in both young and mature hippocampus. In addition, application of NMDA or DHPG is sufficient to induce LTD in both ages. The success of these protocols to induce LTD has allowed us to investigate LTD mechanisms at different developmental stages. Previous studies indicate that AMPAR internalization mediated by GluA2 interactions with NSF and PICK1 is critical for LTD

expression (Malinow and Malenka, 2003; Bredt and Nicoll, 2003; Collingridge et al., 2004; Isaac et al., 2007; Shepherd and Huganir, 2007; Lüscher and Huber, 2010; Collingridge et al., 2010), but it is unknown whether these protein interactions are developmentally regulated. Using the peptide approach, we show that NSF and PICK1 are required for LTD expression in both young and mature mice, indicating that AMPAR internalization serves as a common mechanism as the brain matures. It is important to emphasize that although the peptides (i.e. pep2m and pepEVKI) used are shown to block GluA2 interactions with NSF and PICK1 respectively based on in vitro biochemical assays (Nishimune et al., 1998; Luthi et al., 1999; Daw et al., 2000; Braithwaite et al., 2002; Lee et al., 2002), the effect on other targets in neurons cannot be ruled out. In addition, the effect of these peptides on LTD could be a consequence of depleting/inactivating the NSF and PICK1 protein, and rather than their interactions with GluA2 specifically.

In clear contrast with NSF and PICK1, we show that the involvement of cofilin in LTD expression is developmentally regulated. Thus, in young mice, while the inhibitory cofilin peptide pS3 has no effect on either LFS- or DHPG-induced LTD, it completely blocks both PP-LFS- and DHPG-induced LTD in mature mice. Similar results are obtained for the actin-depolymerization inhibitor phalloidin. Therefore, cofilin-mediated actin depolymerization is essential for LTD expression specifically at mature synapse. These results are consistent with the above biochemical data showing that DHPG-induced cofilin activation is more pronounced in mature than in young mice. It is possible that in young mice, because of the relatively high level of cofilin expression, the effect of cofilin peptides is not as effective as in mature mice, but this is unlikely because increasing the peptide concentration to four times of that used in mature mice, still has no effect on LTD in young mice. It is conceivable that the actin cytoskeleton at this age is already at a depolymerized or dynamic state, and therefore further activation of cofilin is no

longer needed to induce actin reorganization. Alternatively, other actin regulators such as Arp2/3 complex known to be regulated by PICK1 (Rocca et al., 2008; Nakamura et al., 2011; Rocca et al., 2013) may function to mediate actin change at the young synapse. Further experiments will be needed to elucidate how cofilin and Arp2/3 interact to regulate LTD during developmental maturation.

In summary, we have shown that cofilin expression and activation is developmentally regulated and that its involvement in LTD is specific to the mature synapse. Future studies will include identification of actin regulatory mechanisms in young mice and how they change as brain matures. In this context, other effectors targeted by the Rho family small GTPases and associated kinases such as PAKs (Govek et al., 2005).may be important to explore.

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## Figure legend

### **Fig. 1. Cofilin protein level and its activation are developmentally regulated.**

(A, B) Western blot analysis and summary graphs of hippocampal protein lysates showing that total cofilin (A), phosphorylated cofilin (p-cofilin) (A) and p-cofilin/cofilin ratio (B) decrease significantly in mature compared to young mice.



(C) Western blot analysis and summary graphs of hippocampal insoluble protein pellet showing that total cofilin and p-cofilin decrease significantly in mature compared to young mice.

(D, E) Western blot analysis and summary graphs of hippocampal synaptosomal fractions showing that total cofilin (D), p-cofilin (D) and p-cofilin/cofilin ratio (E) decrease significantly in mature compared to young mice.

(F, G) Western blot analysis and summary graphs of hippocampal protein lysates (F) and synaptosomal fractions (G) showing no differences in ADF protein level between young and mature mice.

(H) Western blot analysis and summary graphs of hippocampal protein lysates showing that application of DHPG (100  $\mu$ M, 10 min) decreases p-cofilin, but not total cofilin in young mice.

(I) Western blot analysis and summary graphs of hippocampal protein lysates showing that application of DHPG decreases p-cofilin, but not total cofilin in mature mice.

(J) Western blot analysis and summary graphs of hippocampal protein lysates showing that application of DHPG decreases the p-cofilin/cofilin ratio more in mature than in young mice.

**Fig. 2. LTD can be induced in both young and mature hippocampus using different protocols.**

(A) LFS induces LTD in young, but not in mature mice.

(B) PP-LFS induces LTD in both young and mature mice.

(C) Application of NMDA (25  $\mu$ M, 3 min) induces LTD in both young and mature mice.

(D) Application of DHPG (100  $\mu$ M, 10 min) induces LTD in both young and mature mice.

In this and all other figures of electrophysiological experiments, the representative traces are averages of six successive responses at the indicated time points. Scale bars: 0.5 mV/10 ms for field recordings and 50 pA/50 ms for whole-cell recordings.

**Fig. 3. LTD requires NSF in both young and mature mice.**

- (A) The control NSF peptide, pep4c, has no effect on either basal synaptic responses (Ctrl) or LFS-induced LTD (LFS) in young mice.
- (B) Pep4c has no effect on either basal synaptic responses or PP-LFS-induced LTD in mature mice.
- (C) The active NSF peptide, pep2m, alone or plus LFS depresses synaptic responses to a same degree in young mice.
- (D) Pep2m alone or plus PP-LFS depresses synaptic responses to a same degree in mature mice.
- (E) Pep2m occludes PP-LFS-LTD in mature mice.

**Fig. 4. PICK1 is required for LTD in both young and mature mice.**

- (A) The control PICK1 peptide, pepSVKE, has no effect on either basal synaptic responses or LFS-induced LTD in young mice.
- (B) PepSVKE peptide has no effect on either basal synaptic responses or PP-LFS-induced LTD in mature mice.
- (C) The active PICK1 peptide, pepEVKI, inhibits LFS-induced LTD without affecting basal synaptic responses in young mice.
- (D) PepEVKI inhibits PP-LFS-induced LTD without affecting basal synaptic responses in mature mice.

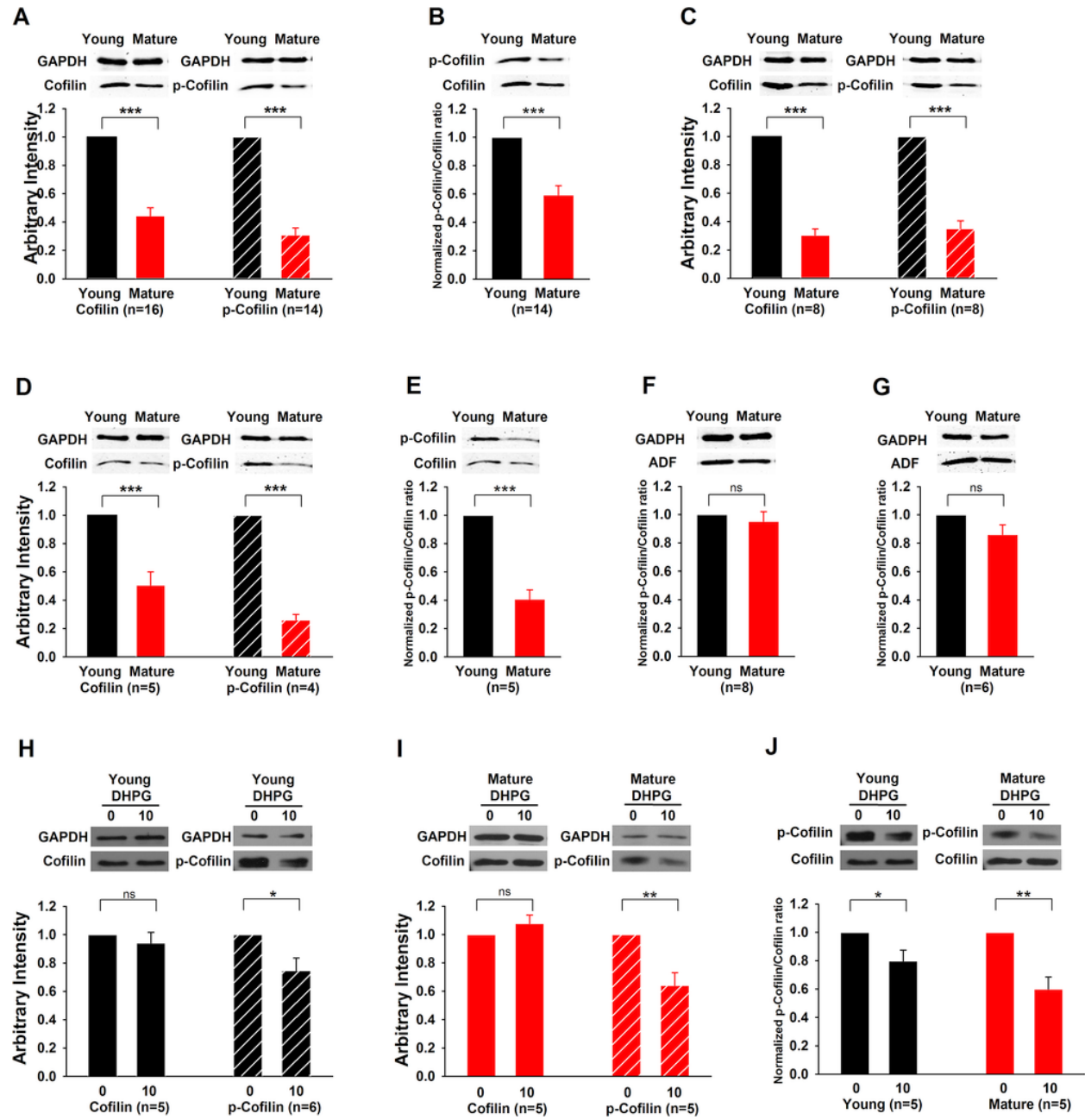
**Fig. 5. Synaptically induced LTD requires cofilin-mediated reorganization in mature, but not in young mice.**

- (A) Phalloidin (100  $\mu$ M) has no effect on LFS-induced LTD in young mice.
- (B) Phalloidin (100  $\mu$ M) has no effect on PP-LFS-induced LTD in young mice.
- (C) Phalloidin (100  $\mu$ M) blocks PP-LFS-induced LTD in mature mice.
- (D) Cofilin inhibitory peptide pS3 (200  $\mu$ M) or enhancing peptide S3 (200  $\mu$ M) has no effect on LFS-induced LTD in young mice.
- (E) Cofilin inhibitory peptide pS3 at 200  $\mu$ M (pS3) or 800  $\mu$ M (high pS3) or enhancing peptide S3 has no effect on PP-LFS-induced LTD in young mice.
- (F) Cofilin inhibitory peptide pS3 (200  $\mu$ M) but not enhancing peptide S3 (200  $\mu$ M) blocks PP-LFS-induced LTD in mature mice.

**Fig. 6. DHPG-induced LTD requires cofilin-mediated actin reorganization in mature, but not in young mice.**

- (A) Neither cofilin inhibitory peptide pS3 (200  $\mu$ M) or enhancing peptide S3 (200  $\mu$ M) has any effect on DHPG-induced LTD in young mice.
- (B) Peptide pS3, but not peptide S3, blocks DHPG-induced LTD in mature mice.
- (C, D) pepEVKI, but not pepSVKE, inhibits DHPG-induced LTD in both young (C) and mature (D) mice.

**Fig. 1**



**Fig. 2**

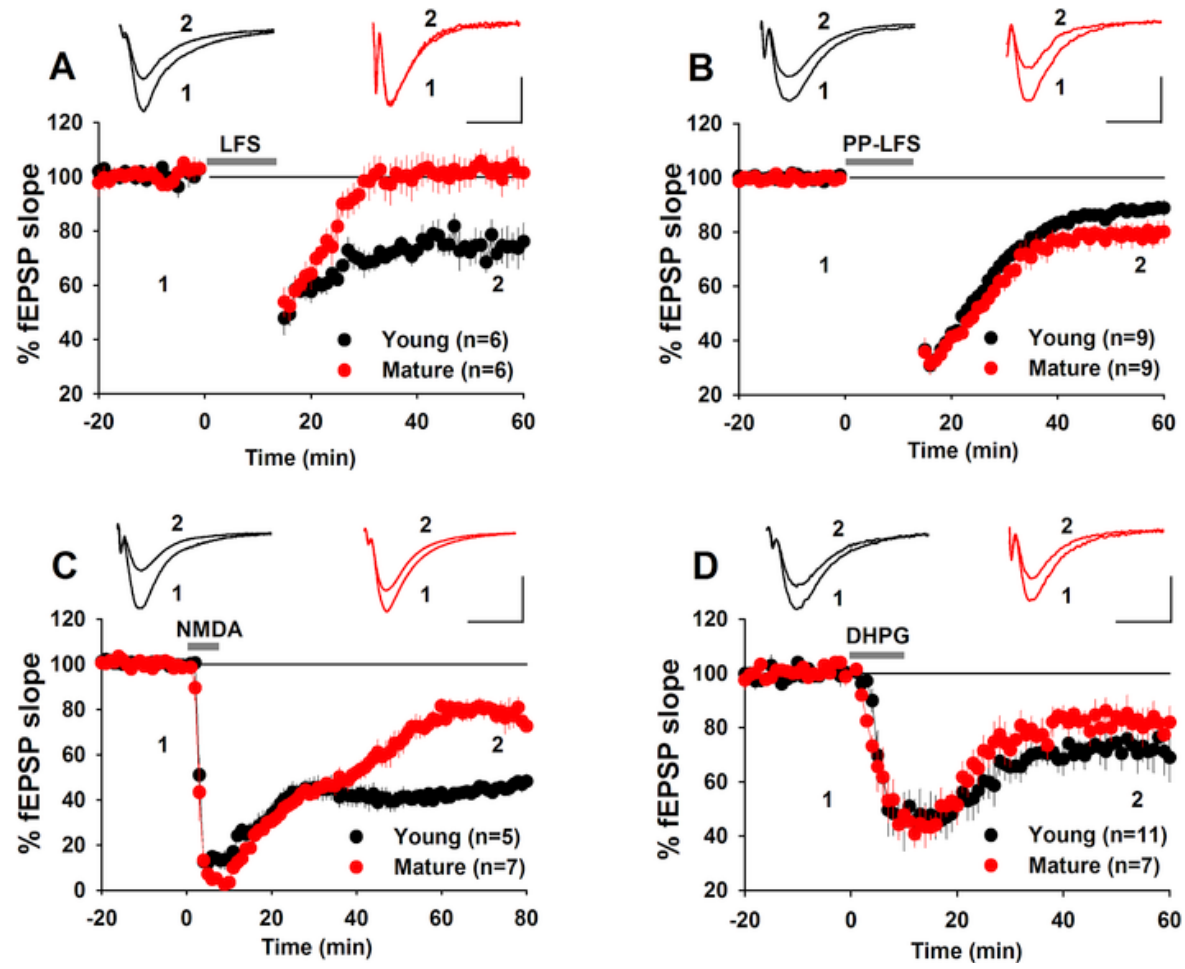
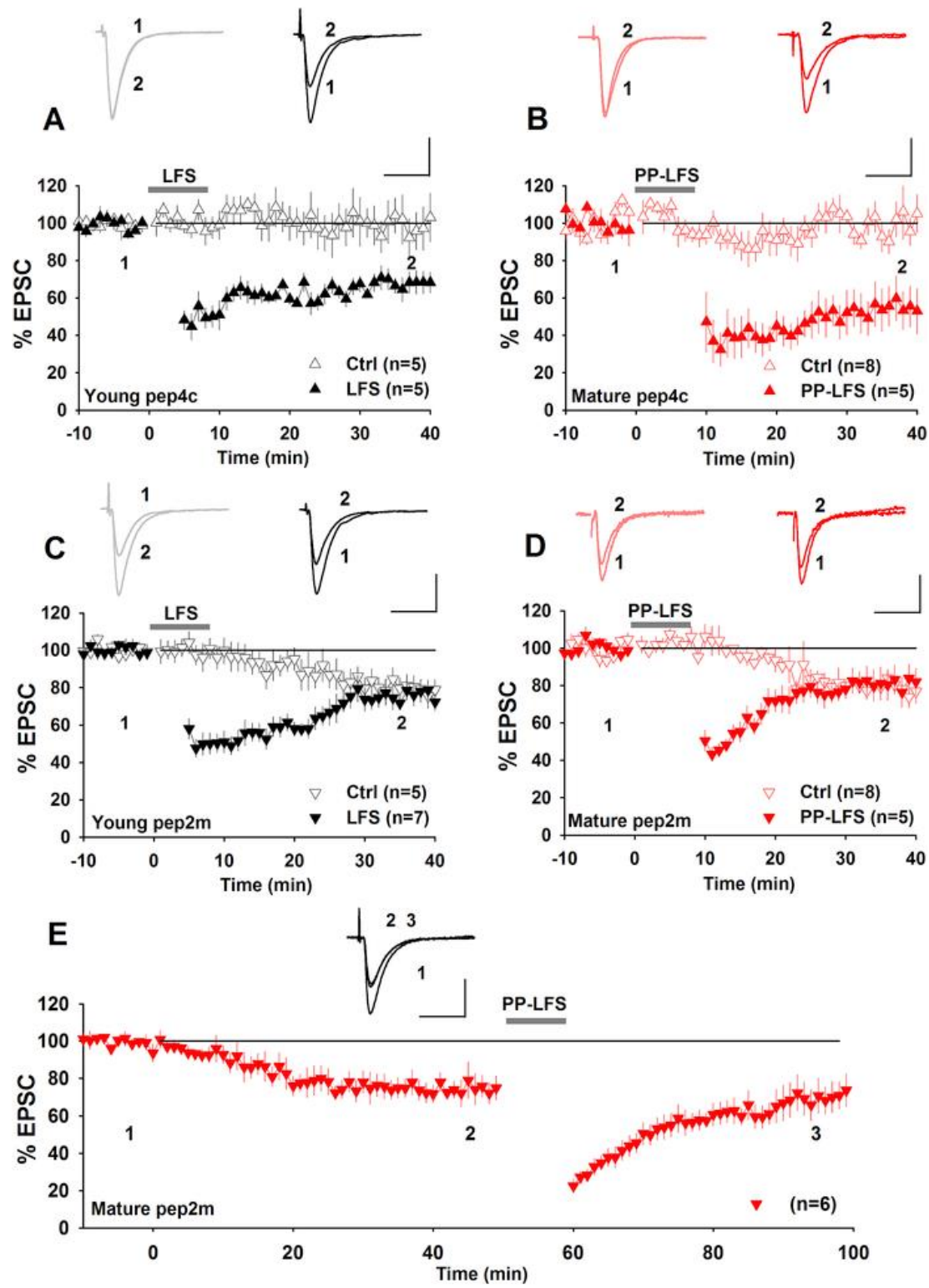
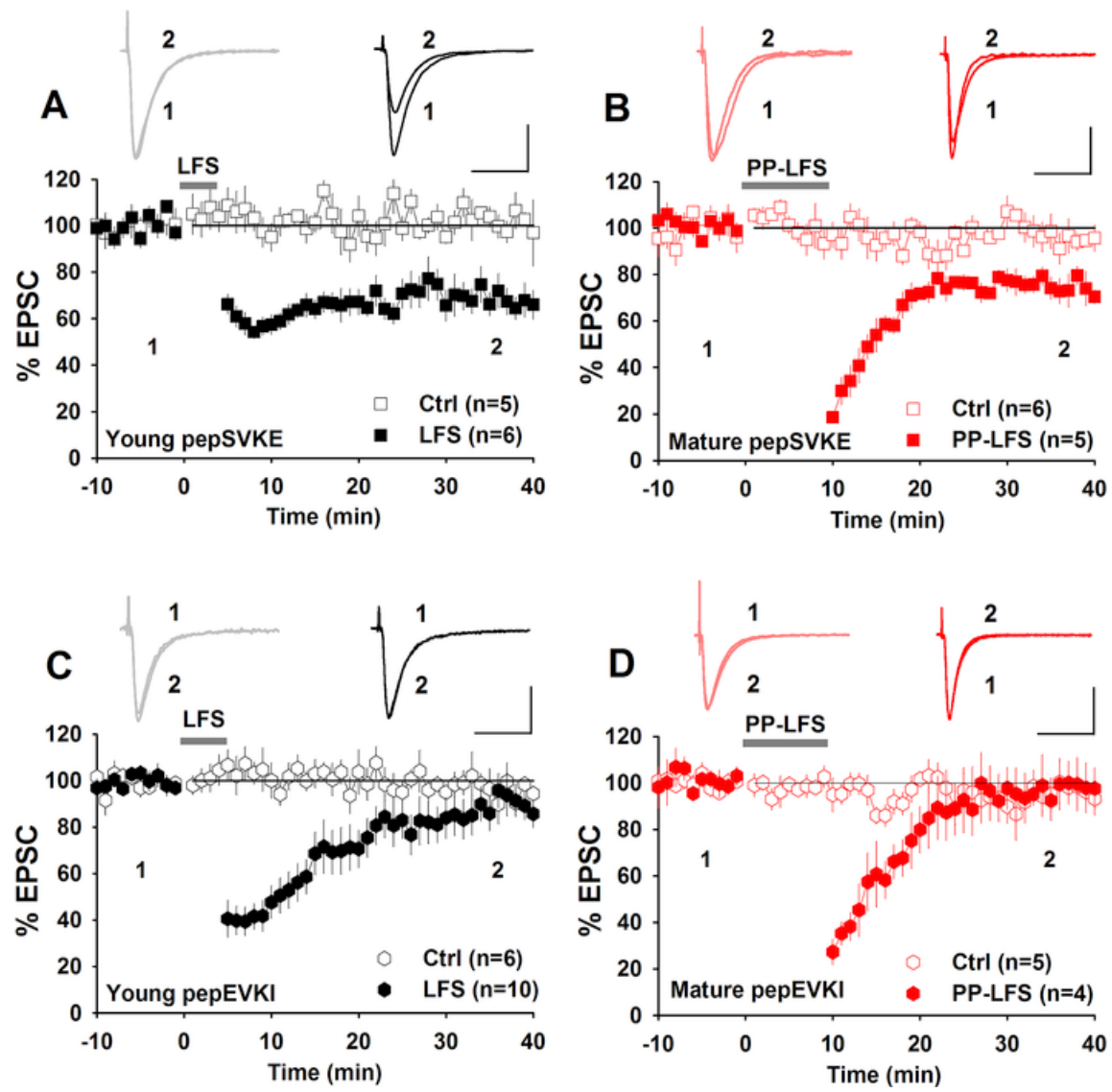


Fig. 3



**Fig. 4**



**Fig. 5**

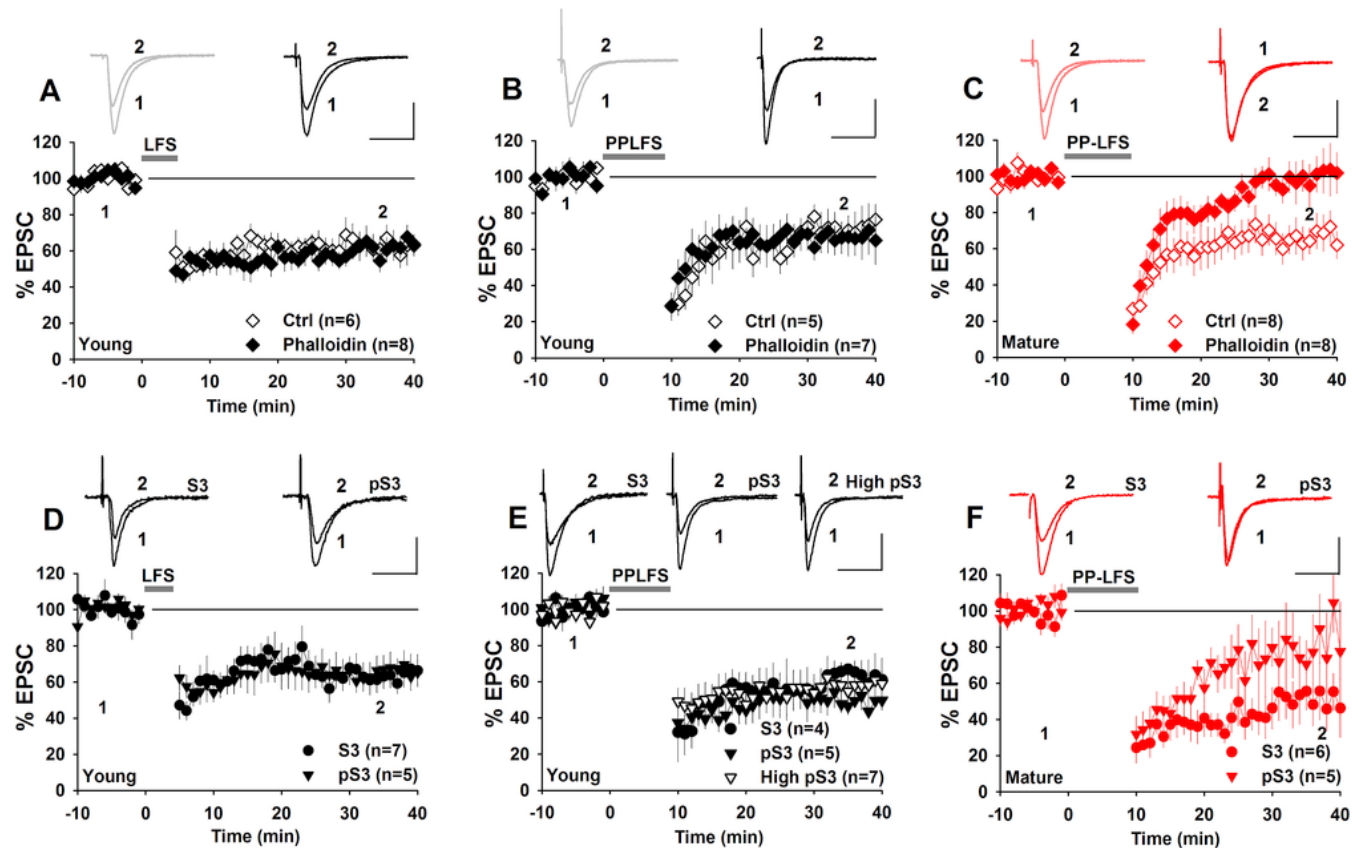




Fig. 6

